

CHARACTERIZATION OF THE KERATAN SULPHATE PROTEOGLYCAN
FROM BOVINE CORNEAL STROMA

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SUMMARY

The keratan sulphate proteoglycans which can be prepared from bovine corneal stroma (I. Axelsson & D. Heinegård, 1975, Biochem. J. 145, 491-500) were characterized by gel chromatography, gel electrophoresis and analytical ultracentrifugation in associative and dissociative solvents. The proteoglycans formed aggregates under associative conditions. The weight average molecular weight was 72000 in dissociative solvent. The presence of S-S-bridges was established. Keratan sulphate linkage regions and oligosaccharides were isolated after proteolysis and analysed. A tentative model for corneal keratan sulphate proteoglycans is suggested.

INTRODUCTION

About one third of the proteoglycans obtained by extraction of bovine corneal slices with 4 M guanidinium chloride can be obtained in a fraction which contains no galactosaminoglycans but a large proportion of the keratan sulphate of the tissue and small amounts of oligosaccharides (Axelsson & Heinegård, 1975). Such a keratan sulphate proteoglycan is unique for cornea. In cartilage and similar tissues all keratan sulphate is attached to the protein core of the cartilage type of proteoglycan which also contains chondroitin sulphate. Therefore, it was considered important to study some of the properties of the unique keratan sulphate proteoglycan prepared from cornea. A preliminary account for some data has been given earlier (Axelsson & Heinegård, 1976).

EXPERIMENTAL

Materials

Corneas from adult cows were prepared as described earlier (Axelsson & Heinegård, 1975). DEAE-cellulose (DE-23) and ecteola-cellulose (ET-11) were from Whatman Ltd., Springfield Mill, Maidstone, Kent, U.K. Dowex ion exchange resins were from Biorad Laboratories, München, G.F.R. Sepharose and Sephadex gels were from Pharmacia Fine Chemicals, Uppsala, Sweden. Papain (twice crystallized) was from Sigma Chemical Co., St. Louis, Mo., U.S.A. Solutions of guanidinium chloride (practical grade; Sigma) were shaken with active charcoal over night and filtered

through filter paper before use to remove UV-absorbing material. High molecular weight hyaluronate from rooster comb was a gift from dr Endre A. Balazs, Eye Research Institute, Columbia University, New York, U.S.A.

Analytical methods.

Column effluents. The effluents from gel columns were analysed for hexose and protein contents by automated versions (Heinegård, 1973) of the methods of Goa (1955) and Lowry et al. (1951) except when guanidinium chloride buffers were used for elution. In these cases, hexose was determined by the manual procedure described by Goa and protein by spectrophotometry at 280 nm.

Neutral sugar. The neutral sugar composition of samples was determined by g.l.c. of their alditol acetates as described elsewhere (Axelsson & Heinegård, 1975). It is critical that the transfer with methanol of the purified alditols to small test tubes prior to acetylation is repeated four times in order to remove the boric acid formed by borate ions during the preceding ion exchange chromatography (Albersheim et al., 1967)

Uronic acids. The oligosaccharide peptides were analysed for uronic acid contents by using the carbazole procedure of Bitter and Muir (1962).

Amino acids and hexosamine. Amino acids and hexosamine were determined on an automatic amino acid analyser after hydrolysis of samples under argon in 6 M-HCl at 110°C for 24 h and in 8 M-HCl at 95°C for 3 h, respectively. Norleucine was used as internal standard for amino acid analysis.

Cysteine and cystine. The total amount of cysteine and cystine was determined as cysteic acid by using the amino acid analyser after oxidation of samples with performic acid described by Moore (1963) and subsequent hydrolysis as described above. Cysteine (SH groups) was determined by the colorimetric reaction described by Grassetti and Murray (1967). Reduced glutathion was used as standard; it gave absorbtivity values close to those described by Grassetti and Murray.

Isolation of keratan sulphate proteoglycans.

Keratan sulphate proteoglycans were extracted with 4 M-guanidinium chloride (pH 5.8), purified by DEAE-cellulose chromatography and fractionated by ethanol precipitation as described earlier (Antonopoulus et al., 1974; Axelsson & Heinegård, 1975). This preparation (referred to as 70P; Axelsson & Heinegård, 1975) was in some cases further purified by gel chromatography in denaturing solvent but was usually used without further purification.

Isolation of keratan sulphate peptides and oligosaccharides from proteoglycan preparations

A portion (25 mg) of the proteoglycan preparation was dissolved in 3 ml 0.1 M-sodium phosphate buffer (pH 7.0) containing 5 mM EDTA and 5 mM cysteine hydrochloride. Papain (0.4 mg) was added and the sample was incubated at 65°C for 4 h. A papain blank was prepared by incubating a solution containing no sample under identical conditions. After incubation the samples and the blank were diluted with 3 ml of water and applied to ecteola-cellulose columns (Cl⁻-form, bed size 1.3 cm x 6 cm) equilibrated with water. About 95 % of the oligosaccharide-peptides were eluted with two bed volumes of water and about 5 % with two bed volumes of 0.02 M HCl; these two oligosaccharide-peptide fractions, which contained all the oligosaccharides (Anseth, 1961) and, in addition, free amino acids and oligopeptides, were pooled. The keratan sulphate-peptides were eluted from the ion exchange resin with 6 M-HCl (Anseth, 1961; Antonopoulos et al., 1967) and a portion was hydrolysed for amino acid analysis. The values were corrected for the small amounts of amino acids found in the corresponding fraction from the papain blank column. The oligosaccharide-peptide fraction was freeze-dried, dissolved in 1.5 ml of a pyridinium acetate buffer (0.25 M pyridin solution with acetic acid to pH 6.5) and applied to a column of Sephadex G-50 superfine (Fig. 12) eluted with the same

pyridinium acetate buffer. Aliquots (0.2 ml) from each fraction were freeze-dried, redissolved in 0.5 ml water and analysed with the Folin and anthrone methods. The anthrone-positive fractions were pooled as indicated in Fig. 12, freeze-dried and analysed for contents of amino acids, hexosamines and neutral sugars.

Reduction and alkylation of proteoglycans

Reduction of proteoglycan samples was performed with dithiothreitol and alkylation with iodoacetic acid as described by Sajdera and Hascall (1969) with minor modifications. Samples (5-6 mg/ml) were dissolved in 4 M-guanidinium chloride/0.1 M-TrisHCl buffer (pH 8.5) and 1 M-dithiothreitol (10 μ l/ml solution) was added. The samples were incubated in a 37°C water bath for 4 h. Then, 1 M-iodoacetic acid (40 μ l/ml solution) was added and the samples were stirred with a magnetic stirrer in darkness at room temperature over night. They were then dialysed at 4°C against the buffer used in the gel chromatography that followed.

Gel chromatography.

Details concerning gel chromatography experiments are given in the text and in figure legends. The methods used for calibration of gels with well-characterized proteins are described elsewhere (Axelsson, 1977). When the elution buffer was changed, at least two bed volumes of the new buffer were passed through the column before the sample

was applied. Buffers containing guanidinium chloride or SDS will be referred to as dissociative solvents since guanidinium chloride and SDS break non-covalent bonds and dissociate e.g. cartilage proteoglycan aggregates. Other buffers will be referred to as associative buffers. The columns packed in and equilibrated with dissociative buffers were never eluted with associative buffers and vice versa. Most associative buffers contained 0.02 % NaN_3 as a bacteriostatic agent. Control chromatographic runs without NaN_3 showed that NaN_3 does not affect the gel chromatographic pattern of the proteoglycans at neutral or acid pH.

SDS-polyacrylamide gel electrophoresis.

Electrophoresis was performed on 7 % polyacrylamide gels in 0.1 % SDS as described by Neville (1971). The gels were stained with coomassie brilliant blue as described by Hascall & Heinegård, (1974). The absorbance at 605 nm was scanned using a Gilford model 222 photometer equipped with a Beckman model DU monochromator, a gel scanning device (constructed by dr Sven Björnsson and Mr Runo Svensson from this laboratory) and a Servogor recorder.

Determination of partial specific volume.

The partial specific volume of the proteoglycans was determined. This required estimation of the dry weights of the proteoglycan samples. Attempts to determine the

dry weight by drying sample portions in vacuum over P_2O_5 followed by weighing at atmospheric pressure were unsuccessful since the sample weight increased rapidly during the first five minutes due to water uptake by the sample as described by Laurent & Anseth (1961). The dry weight determinations were therefore performed with a Cahn RG electrobalance (Ventron Instruments Corp., Paramount, California, U.S.A.) with the weighing device inside a high vacuum system. The air was evacuated by means of a Speedivac high vacuum pump (model ES 50) and a speedivac oil vapour diffusion pump (model E 02). The pressure was monitored with a Penning Gauge Head (model 6). The pumps and the manometer were from Edwards high vacuum Ltd, Crawley, Sussex, U.K. The weight was continually registered by means of a Servogor recorder. The air pressure was maintained at 10^{-5} mm Hg (10^{-3} P) for several days until the weight of the sample was constant. This weight was considered to be the dry weight of the sample. When air was allowed to enter the system, the sample weight increased with 5-10 % within 15 minutes. The sample was utilized for preparation of solutions with known proteoglycan concentration in 0.15 M-NaCl/5mM-TrisHCl (pH 7.0). Corrections were made for the small amounts (1-5 %) of sample that were not dissolved in the buffer. The densities of the solutions were determined by means of a mechanical oscillator (Precision density meter DMA 02C from Anton Paar K.G., Graz, Austria) and the partial

specific volume of the sample was calculated (Kratky et al., 1973).

Molecular weight determination.

The weight average molecular weight of 70P was determined by long-column meniscus depletion sedimentation equilibrium centrifugation (Chervenka, 1970). Three solutions with different sample concentrations were centrifuged at two different speeds in 12 mm double-sector capillary synthetic boundary cells in an An-D rotor in a Beckman-Spinco model E centrifuge. Photographs of the interference optics patterns were analysed in a Nikon comparator and the apparent average molecular weights were calculated from the plots of log fringe displacement versus the square of the radial distance (Chervenka, 1969). No corrections were made for the effects of the solvent (6 M-guanidinium chloride, pH 7) on partial specific volume or molecular weight (Bryce & Crichton, 1971).

Sedimentation velocity ultracentrifugations.

Sedimentation velocity centrifugations were made with an An-D rotor in a Beckman-Spinco model E centrifuge or with an MSE analytical rotor in an MSE Centriscan 75 centrifuge. Schlieren patterns were recorded in both cases. Sedimentation coefficients ($s_{20,w}$) were calculated as described by Schachman (1957).

Polydispersity analysis by transport centrifugations.

Polydispersity of proteoglycans was studied by using the transport centrifugation method described by Pita and Müller (1973). A Sorvall OTD-2 centrifuge and a Beckman SW 50.1 swinging bucket rotor equipped with the adapters described by Pita and Müller were used. Centrifugation was done at 20°C for various times and rotor speeds. The volume of the sample solution was 0.55 ml and 20 fractions, 25 µl each, were aspirated from the top of the tube after centrifugation. The distance from rotor centrum to the meniscus was 6.95 cm. The length of the part of the liquid column that was fractionated for analysis was 3.00 cm. The fractions were analysed for protein (absorbance at 280 nm) in a Zeiss PMQ II spectrophotometer fitted with a 50 µl microcuvette (light path 1.0 cm; J.C. Pita, personal communication).

RESULTS AND DISCUSSION

Characterization of proteoglycans by gel chromatography.

The corneal keratan sulphate proteoglycans (70P) are separated in two main peaks called 70PA and 70PB (Axelson & Heinegård, 1975) when chromatographed on 4 % agarose at neutral pH. The proportions of peak A and B show some variation between different batches of proteoglycans even if they are prepared in the same way. In most preparations there is a slight excess of B over A. The K_d values of the two peaks are always the same. Some material is eluted in the void volume. When the pH of the elution buffer is lowered, the relative size of peak A increases while peak B decreases (Fig. 1). It is possible then that the molecules eluting in peak B change their conformation and/or form aggregates. The amounts of material completely excluded from the gel show only a minor increase. The anthrone profiles (not shown in figure) always follow the Folin curves very closely. Therefore, the hexose/protein ratio is similar in all three peaks. When the samples are pre-incubated in elution buffer for 24 h before chromatography they show the same elution pattern as samples which were not pre-incubated, suggesting that the chromatograms represent equilibrium or near-equilibrium. The changes in the gel chromatographic behavior are to a large extent reversible (dashed curve in Fig. 1). The materials in peaks A and B were isolated from a preparative Sepharose 4B column and rechromatographed on an analytical Sepharose 4B column.

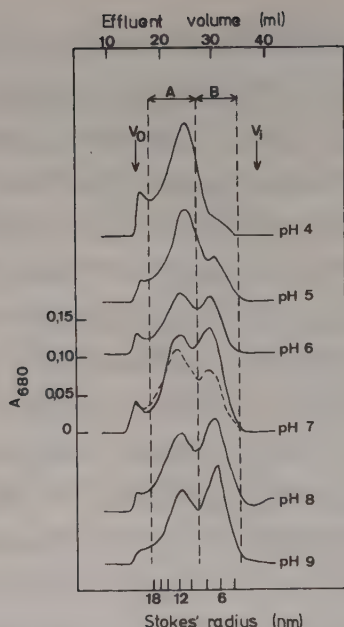


Fig. 1. Chromatography of keratan sulphate proteoglycans on Sepharose 4B at different pH.

Samples (0.25-0.30 mg) were dissolved in 200 μ l elution buffer and applied to the column. The curves show the protein concentration analysed with the Folin method. — — —, Folin absorbance for a sample incubated in the pH 4 buffer for 24 h, then dialysed against pH 7 buffer for 24 h and finally run on the column at pH 7. Elution buffers were 0.6 M-NaCl/0.05 M-acetic acid buffered with sodium hydroxide to pH 4, 5, 6 and 7, respectively, and 0.6 M-NaCl/0.05 M-barbitone sodium buffered with HCl to pH 8 and 9, respectively. The scale showing Stokes' radius was obtained by calibration with globular proteins. The bed size of the column was 0.6 cm x 134 cm and the fraction size 0.57 ml.

They chromatographed as two separate peaks (Fig. 2a,b) with the same elution volume as the original peaks. It is most likely then, that the material in peak A either represents aggregated peak B molecules or that they are unrelated molecular species. It is less likely that peak A only contains molecules of the same primary structure as the peak B molecules, but with a different conformation.

When the material in 70PB is separately chromatographed at the lower pH however, the peak is eluted at essentially the same position as at pH 7 (Fig. 2c). Since no peak was eluted at this position when 70P was chromatographed at acid pH, the simplest explanation for the chromatographic behavior of 70P and isolated 70PB is that the molecules in 70PA can interact with the molecules in 70PB to form aggregates.

The effects of different salt concentrations and of dissociating agents on the gel chromatographic pattern of the proteoglycans were tested. The same elution patterns were obtained with 0.15, 0.3, 0.6 and 0.9 M-NaCl in 0.05 M-sodium acetate buffer (pH 7.0) (Axelsson, 1977). On a Sepharose 4B column eluted with 4 M-guanidinium chloride most of the proteoglycans were eluted in a peak with a K_d value of about 0.63 (Fig. 3a), i.e. close to that of peak B (0.61) eluted with associative buffers from Sepharose 4B. The effluent was pooled as indicated in Fig. 3a, recovered and rechromatographed under associative conditions (Fig. 3b). The elution curves at pH 7 and 4 are very similar to the original patterns (cf Fig. 1). It is then

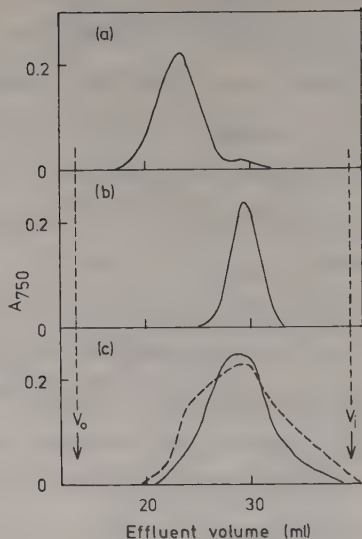


Fig. 2. Rechromatography on Sepharose 4B of fractions A and
 70PA and 70PB were isolated from a run of 70P on a prepara-
 tive Sepharose 4B column (bed size 1.6 cm x 128 cm; fraction
 size 4.6 ml) eluted with 0.5 M-sodium acetate (pH 7.0), dia-
 lysed against water, freeze-dried and portions (about 0.8 mg)
 were applied to an analytical Sepharose 4B column (bed size
 0.6 cm x 134 cm; fraction size 0.84 ml) eluted with the same
 buffer. The effluent was analysed for protein (Folin). (a)
 70PA; (b) 70PB. (c) Chromatography on the same column of
 another preparation of 70 PB isolated with the same proce-
 dure. Elution was made with 0.15 M-NaCl/5mM barbitone
 sodium buffer/0.02 % NaN_3 (pH 7.0) (———) or 0.15
 M-NaCl/5mM sodium acetate buffer/0.02 % NaN_3 (pH 4.0)
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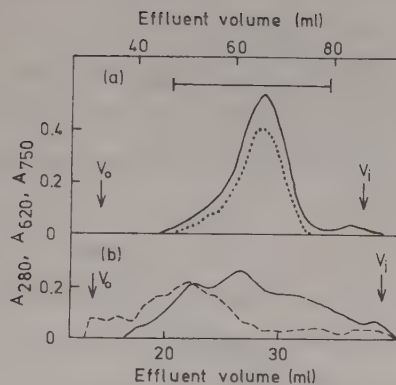


Fig. 3. Chromatography of proteoglycans on Sepharose 4B under dissociative and associative conditions.

(a) A portion (20 mg) of 70P was applied to a Sepharose 4B column (bed size: 0.8 cm x 150 cm) eluted with 4 M-guanidinium chloride/0.05 M-sodium acetate (pH 5.8). ———, Protein (absorbance at 280 nm); ····, hexose (anthrone absorbance at 620 nm). (b) Rechromatography on a Sepharose 4B column (bed size 0.6 cm x 134 cm) of the material indicated by the horizontal bar in Fig. 3a. The material was first dialysed against water and freeze-dried: ———, Protein (Folin absorbance at 750 nm) curve from 1.15 mg sample eluted with 0.15 M-NaCl/5mM barbitone sodium buffer/0.02 % NaN_3 (pH 7.0); -----, protein (Folin) curve from 0.85 mg sample eluted with 0.15 M-NaCl/5mM sodium acetate buffer/0.02 % NaN_3 (pH 4.0).

likely that gel chromatography in 4M-guanidinium chloride does not remove any extraneous protein which can cause aggregation. When the proteoglycans were chromatographed on a Sepharose 4B column equilibrated with 1 % SDS (pH 7.0) they also showed one major retarded peak with $K_d = 0.57$ (Fig. 4a) i.e. a slightly lower K_d value than that of 70PB eluted with associative buffer. SDS will bind to proteins to a much larger extent than guanidinium chloride and unfolded proteins show partial "stiff rod" properties in SDS solutions but almost pure "random coil" properties in guanidinium chloride solutions (Reynolds & Tanford, 1970; Nozaki et al., 1976). It is therefore possible that solutions of SDS cause such conformational changes in the proteoglycans that 70PB molecules are eluted with a larger molecular size because of a partial "stiff rod" character. Rechromatography on a Sepharose 4B/associative buffer column of the two fractions isolated from the Sepharose 4B/SDS buffer column is shown in Fig. 4c,d. Both fractions are eluted in the region of 70PA (K_d : 0.32-0.40; K_d for 70PA is about 0.36 in 0.6 M NaCl, pH 7) both at pH 4 and 7. It may be that the remaining, tightly bound SDS cannot dissociate proteoglycans but may cause a conformational change that facilitates aggregation of proteoglycans. Alternatively same conformational change may be caused by low pH or reduction and alkylation. Less likely, the SDS may directly serve as a link between proteoglycan molecules.

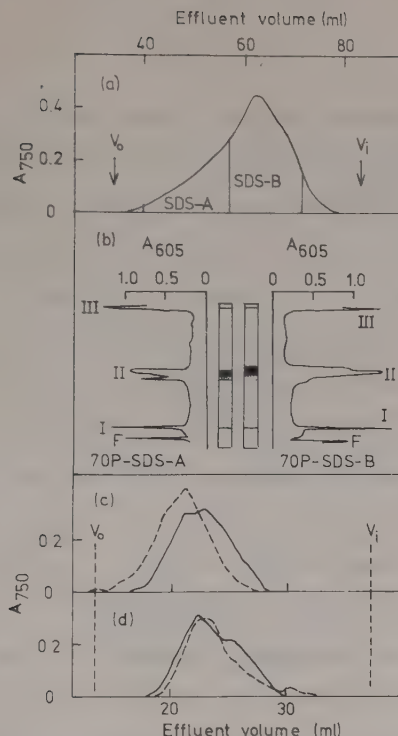


Fig. 4. Gel chromatography and gel electrophoresis of proteoglycan-SDS complex.

(a) Chromatography of 70P (18 mg) on a Sepharose CL-4B column (bed size 0.9 cm x 141 cm; fraction size 1.1 ml) eluted with 1 % (w/v) SDS in 5 mM-barbitone sodium buffer (pH 7.0) after preincubation of the sample at 37°C for 2 h in this solvent. The effluent was analysed for protein by the Folin method. The indicated fractions 70P-SDS-A and 70P-SDS-B were dialysed against water and freeze-dried. (b) SDS-gel electrophoresis of 70P-SDS-A and 70P-SDS-B. Peak F (front) was caused by a steel needle inserted at the position of bromophenol blue which was included in the samples. (c) - (d) Rechromatography of portions (0.7 - 0.9 mg) of 70P-SDS-A and 70P-SDS-B on a Sepharose 4B column (bed size 0.6 cm x 134 cm; fraction size 0.58 ml) eluted with 0.15 M-NaCl/5 mM-barbitone sodium/0.02 % NaN_3 (pH 7.0) or with 0.15 M-NaCl/5 mM-sodium acetate/0.02 % NaN_3 (pH 4.0) and analysed for protein by the Folin procedure. (c) 70P-SDS-A eluted at pH 7 (—) and pH 4 (---); (d) 70P-SDS-B eluted at pH 7 (—) and pH 4 (---).

The effects observed cannot be attributed to an effect of SDS or guanidinium chloride on the Sepharose gel since such solutions do not change the properties of Sepharose 4B (Fish et al., 1970). The various batches of Sepharose 4B used in the present investigation showed negligible differences in chromatographic properties.

The proteoglycan fractions isolated from the Sepharose 4B/SDS buffer column (Fig. 4a) are heterogenous as indicated by SDS-polyacrylamide gel electrophoresis (Fig. 4b). Most of the material migrates with the intermediate (II) fraction which contains more than one component. No attempt was made to characterize the subfractions.

Reduction and alkylation of the proteoglycans gave a drastic decrease in polydispersity; all reduced and alkylated proteoglycans eluted at the position of peak A in associative buffers both at pH 7 and pH 4 (Fig. 5). With 6M-guanidinium chloride (pH 4 and 7) as eluant the reduced and alkylated proteoglycans showed the same retarded elution pattern as in Fig. 3a. These results indicate that the conformation of the protein core in the proteoglycans is important. The native conformation which probably is more ordered only allows interaction at acid pH. Unfolding the protein by reduction may expose charged groups and it is possible that some of the interactions studied are of electrostatic nature and perhaps nonspecific. A series of experiments were therefore made to test interactions of 70P with other proteoglycans and glycosamino-

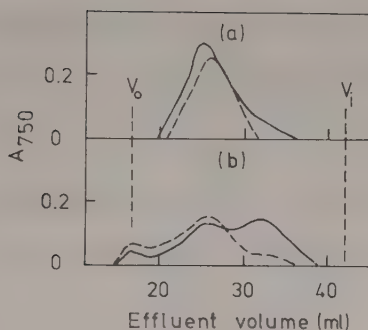


Fig. 5. Gel chromatography of reduced and alkylated proteoglycan.

A Sepharose 4B column (bed size 0.6 cm x 134 cm; fraction size 0.98 ml) was eluted with 0.6 M-NaCl/0.05 M-sodium acetate buffer, pH 4.0 and pH 7.0, respectively, and the effluent was analysed for protein by the Folin procedure. The same preparation of 70P was used for all experiments.

(a) Reduced and alkylated 70P chromatographed at pH 7 (—) and pH 4 (---). (b) Untreated control chromatographed at pH 7 (—) and pH 4 (---). The control sample was incubated in the same guanidinium chloride solution as the other sample but no reducing or alkylating agents were added.

glycans. Portions (2 mg) of 70P were dissolved in 1 ml 4 M-guanidinium chloride/0.05 M-sodium acetate (pH 5.8) containing 20 μ g of hyaluronate or 200 μ g of bovine nasal cartilage proteoglycan monomer (Al-D1; Heinegård, 1972) and incubated at room temperature for 3-4 h. Each portion was then divided into two equal fractions that were dialysed against 100 ml 0.15 M-NaCl/5 mM-sodium acetate buffer/0.02 % NaN_3 (pH 4.0) and 0.15 M-NaCl/5 mM-barbitone sodium buffer/0.02 % NaN_3 (pH 7.0), respectively. The samples were chromatographed on Sepharose 4B eluted with the buffers described. A third 2 mg portion of 70P was treated in the same way but without addition of hyaluronate or cartilage proteoglycans to serve as a control. The six chromatograms obtained are shown in Fig. 6. Hyaluronate and Al-D1 are eluted in the void volume of Sepharose 4B. Almost all of the protein in the chromatograms comes from corneal proteoglycans since neither hyaluronate nor Al-D1 contains appreciable amounts of protein. The curves suggest then that a portion of 70P is bound to hyaluronate and Al-D1 at pH 4 but not at pH 7. It is therefore likely as discussed above that at lower pH, positively charged groups in the proteoglycan protein may interact with the negative charges of the glycosaminoglycan chains. The pH-dependent changes in elution pattern discussed may therefore be caused by unspecific proteoglycan-proteoglycan interactions.

The Sepharose 4B column used for the experiments pre-

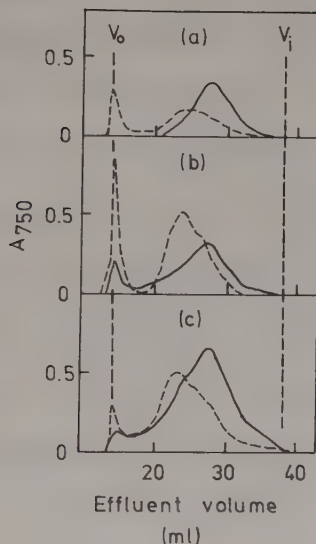


Fig. 6. Gel chromatography of proteoglycans after incubation with hyaluronate and with proteoglycan monomers from bovine nasal cartilage.

The same preparation of 70P was used in all experiments; this preparation contained only small amounts of 70PA at pH 7. A Sepharose 4B column (bed size 0.6 cm x 134 cm; fraction size 0.6 ml) was eluted with 0.15 M-NaCl/5 mM-barbitone sodium buffer/0.02 % NaN_3 (pH 7.0) or 0.15 M-NaCl/5 mM-sodium acetate buffer/0.02 % NaN_3 (pH 4.0)

———, Protein (Folin) curve obtained at pH 7.0;
 ----, protein (Folin) curved obtained at pH 4.0 (a) 70P and hyaluronate; (b) 70P and nasal cartilage proteoglycan monomers; (c) 70P control incubated in guanidinium chloride solution like the other samples but without addition of other macromolecules.

sented in Fig. 1 was calibrated with globular proteins (Axelsson, 1977) in order to determine the average molecular size of 70PA and 70PB. It was found that 70PA and 70PB have the same K_d as proteins with Stokes' radius about 11 nm and 7 nm, respectively. These values are very approximative since the calibration curves was extrapolated for values for Stokes' radius above 8 nm and since K_d is dependent on the shape of the molecules (Nozaki et al., 1976; Axelsson, 1977).

In summary, two populations (70PA and 70PB) of corneal keratan sulphate proteoglycans (70P) can be prepared by chromatography on Sepharose 4B in physiological saline. 70PB is the predominant fraction in most batches of corneal proteoglycans. It has an elution volume between jack bean urease and bovine thyroglobulin (Stokes' radii 6.5 and 8.6 nm, respectively) (Axelsson, 1977), which means that it is much smaller than cartilage proteoglycans. At low pH or after reduction and alkylation it seems to aggregate; this aggregation is facilitated by the presence of 70PA. Most of 70PA is dissociated to monomers with approximately the same K_d as 70PB by SDS and guanidinium chloride. However, other explanations for the relations between 70PA and 70PB are possible. Therefore, further studies comprising ultracentrifugation, determination of S-S-bridges and proteolytic degradation were made.

Characterization of proteoglycans by ultracentrifugation.

Sedimentation velocity runs of 70P in associative buffers gave a very polydisperse pattern. Therefore, 70PA and 70PB were analysed separately in associative buffers (Fig. 7) and 70P was centrifuged only in dissociative buffer (Fig. 8). 70 PB shows a distinct schlieren peak with $s_{20,w} = 2.7$ S (sample concentration 3.6 g/l; the partial specific volume for 70P was found to be 0.67 ml/g; this value was used in the subsequent calculations) (Fig. 7a). The schlieren pattern obtained with 70PA contains a corresponding peak ($s_{20,w} = 3.1$ S; sample concentration 3.6 g/l) but most of the material sedimented faster and showed a large polydispersity (Fig. 7b). In 6 M-guanidinium chloride solution, a single boundary was obtained with 70P (Fig. 8a); the $s_{20,w}^O$ value was 1.7 S for the untreated proteoglycan and 1.5 S for the reduced and alkylated proteoglycan (Fig. 8b). The difference is small but may suggest an unfolding of the proteoglycan by the reduction. Neither sedimentation analysis nor gel chromatography indicated that peptides were released by reduction. It may be concluded from Fig. 8a that there are small amounts of faster sedimenting material even in 6 M-guanidinium chloride, but most of the proteoglycans seem to be dissociated into monomers. Fraction 70PA contains molecules of higher molecular weight than 70PB since 70PA has a larger molecular size (according to gel chromatographic data) and higher average $s_{20,w}$ (according to Fig. 7). Therefore, it is probable that the changes induced in 70PB by e.g. low

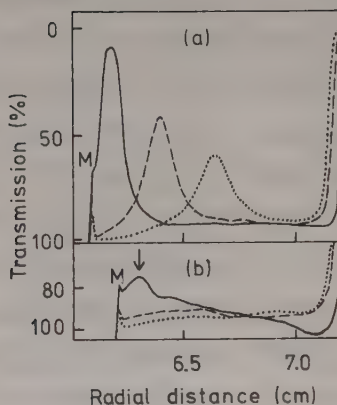


Fig. 7. Sedimentation velocity centrifugation of 70PA and 70PB in associative buffers.

Centrifugation was performed in an MSE Centriscan 75 centrifuge at 50000 rev./ min. The samples (3.6 mg/ml) were dissolved in and dialysed against 0.6 M-NaCl/5 mM-barbitone sodium/0.02 % NaN₃ (pH 7.0). M depicts the meniscus. (a) Schlieren patterns of 70PB obtained 40 s after the curves in (b). $s_{20,w}$ for the main peak was 2.7 S. (b) Schlieren patterns of 70PA obtained 30, 120 and 210 min after reaching full speed. $s_{20,w}$ for the peak indicated by the arrow was 3.1 S.

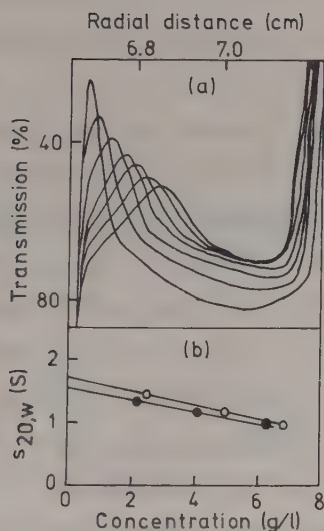


Fig. 8. Sedimentation velocity centrifugation of the proteoglycans (70P) in dissociating buffer.

The samples were dissolved in and dialysed against 6 M-guanidinium chloride/0.05 M-TrisHCl (pH 7.0).

(a) Schlieren curves from a run of untreated 70P in an MSE Centriscan 75 centrifuge at 60000 rev./min. The sample concentration was 4.9 g/l and the curves were obtained 40, 80, 120, 165, 210, 255 and 300 min. after reaching full speed. (b) Determination of sedimentation constants for untreated (○) and reduced and alkylated (●) 70P. By extrapolation of the best least square lines the $s_{20,w}^0$ values 1.7 S and 1.5 S were obtained for untreated and reduced and alkylated 70P, respectively.

pH involves aggregation. Further evidence was obtained by analysis of polydispersity by the transport centrifugation method described by Pita and Müller (1973) (Fig. 9). The protein concentration (measured as A_{280}) at the plateau region (s values 20-30 S) was 70 % of the initial protein concentration both at pH 4 and pH 7. This decrease in protein cannot be fully explained by radial dilution. Therefore, about twenty per cent of the material sedimented so fast that it was not detected under the experimental conditions. Of the remaining material, about 70 % showed apparent s values above 5S at pH 4 while at pH 7 only about 15 % had s values above 5S. These results suggest that low pH causes aggregation of the proteoglycans.

The weight average molecular weight of the proteoglycan monomer was 72000 as determined by sedimentation equilibrium centrifugation at 22000 rev./min with dissociating solvent (Fig. 10). The plots of the logarithm of fringe displacement versus the square of the radial distance were perfectly linear suggesting monodispersity of the sample. However, a considerable polydispersity is suggested by the gel chromatography pattern in guanidinium chloride. Therefore, the sample was centrifuged at a higher speed (28000 rev./min). The plots were again linear but gave a lower molecular weight value (53000). Thus, there is a considerable polydispersity of the proteoglycans despite the linear fringe displacement plots; the same is true for keratan sulphate peptides from cartilage proteoglycans (Heinegård & Axelsson, 1977). It was not possib

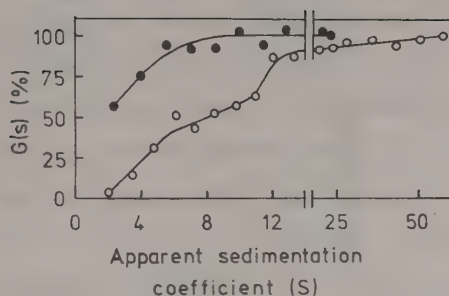


Fig. 9. Polydispersity analysis of proteoglycans (70P) by transport centrifugation in associative buffers.

The fractions were analysed for protein (A_{280}) and $G(s)$ curves were calculated as described by Pita & Müller (1973). A portion of 70P (1.8 g/l) in 0.15 M-NaCl/5 mM-sodium acetate (pH 4.0) was centrifuged at 52950 rev./min for 83.3 min and at 48930 rev./min for 30 min (○). Another portion (1.8 g/l) was centrifuged in 0.15 M-NaCl/5 mM-sodium phosphate (pH 7.0) at 44600 rev./min for 100 min (●). Centrifugation times were measured from the start of acceleration to the start of retardation.

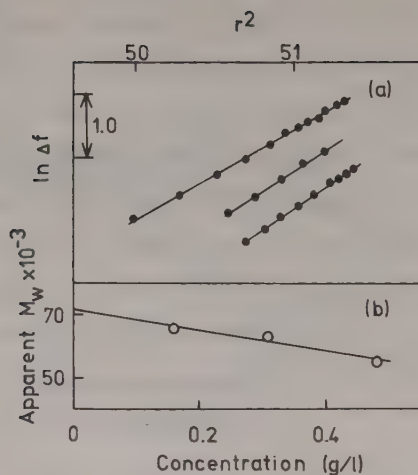


Fig. 10. Molecular weight determination of proteoglycans.

(a) Plot of $\ln$ fringe displacement (Δf) in μm versus the square of the radial distance (r) in cm. The three curves correspond to sample concentrations 0.48 g/l (top curve), 0.32 g/l, and 0.16 g/l (bottom curve) in 6 M-guanidinium chloride/0.05 M-Tris/HCl (pH 7.0). The rotor speed was 22000 rev./min.

(b) Apparent weight average molecular weights (M_w) plotted versus concentration. The best least square line intersects the ordinate at $M_w = 72000$.

le to reach meniscus depletion at speeds significantly lower than 22000 rev./min. We consider 72000 to be the better approximative average molecular weight value for the proteoglycan monomers.

The physical parameters for proteoglycan monomers obtained in this investigation (molecular weight approximately 72000; sedimentation constant 1.7×10^{-13} s; partial specific volume 0.67 ml/g) were used to calculate Stokes' radius (12 nm) and frictional ratio (4.5) by Svedberg's equation (Svedberg & Pedersen, 1940) and equations discussed elsewhere (Axelsson, 1977). The value for Stokes' radius (12 nm) is higher than that obtained by gel chromatography (7 nm; Figs. 1,3). The discrepancy may be explained by experimental errors, e.g. a high average molecular weight value caused by the presence of small amounts of high molecular weight material, or by a change in conformation induced by guanidinium chloride and reflected in the low $s_{20,w}$ values obtained in guanidinium chloride solutions (Fig. 8). It is less likely, however, that such a conformational change would not change the gel chromatographic pattern (cf Fig. 3). Alternatively, the role of asymmetry may be considered (Nozaki et al., 1976; Axelsson, 1977); gel chromatography underestimates Stokes' radius for the monomers if they are asymmetric. The value for frictional ratio (4.5) reflects the high degree of hydration shown by proteoglycans but may also reflect molecular asymmetry.

In summary, data from ultracentrifugation confirm that corneal keratan sulphate proteoglycans consist of a major population of monomers which form aggregates at certain conditions. There is also evidence for a small population of larger proteoglycans that do not dissociate readily. (In fact, gel chromatographic data (Fig. 2c) suggest that these large molecules are necessary for a high degree of aggregation of monomers.) Very approximative values for average molecular weight and Stokes' radius of the monomers are 72000 and 12 nm, respectively. Proteoglycan monomers from bovine hyaline cartilage are much larger and have average molecular weights of $2-3 \times 10^6$ (Hascall & Sajdera, 1970) and average molecular lengths around 300 nm (Thyberg et al., 1975). Thus, the size of corneal keratan sulphate proteoglycan monomers is only about 3-7 % of the size of cartilage proteoglycan monomers.

The corneal proteoglycan monomers, like cartilage proteoglycan monomers, are much less compact than most globular proteins. Calf thyroglobulin, for example, has a molecular weight (669000) ten times that of the proteoglycan monomers but its Stokes' radius (8.6 nm) is smaller (data from Edelhoch, 1960).

Demonstration of S-S-bridges.

The effect of reduction on the properties of the proteoglycans (Figs. 5, 8) suggests that S-S-bridges are important for the three-dimensional structure of

the proteoglycans. The existence of S-S-bridges (cystine) and the absence of SH-groups (cysteine) were confirmed by chemical analysis (Table 1). If the minimum molecular weight of 27000 per cystine in Table 1 is compared with the average molecular weight value (72000) it may be concluded that the proteoglycans contain an average of two to three S-S-bridges per molecule. Reduction did not remove any small peptides or cleave the molecule as revealed by gel chromatography or analytical ultracentrifugation. It is probable, then, that the S-S-bridges connect different parts of the same polypeptide chain. Reduction of the proteoglycans results in changes in conformation and ability to form aggregates (Figs. 5, 8). The most simple explanation is that the molecule is unfolded when reduced. At least one S-S-bridge in the molecule may then connect cysteine residues that are interspaced by several other amino acid residues forming a loop of the peptide chain.

Analysis of isolated fractions 70PA and 70PB did not reveal any significant differences in keratan sulphate chain size (Fig. 11), oligosaccharide content (Fig. 11, legend) or keratan sulphate/protein ratio (Axelsson & Heinegård, 1975). Table 2 shows differences between A and B in cysteine and methionine contents. The yield of these amino acids varies considerably between different analyses because the hydrolysis conditions are not optimal for the sulphur-containing

Table 1. Cysteine and cystine contents of the proteoglycans.

The buffers used were Krebs-Ringer phosphate buffer (pH 7.4; Grassetti & Murray, 1967); 0.15 M-NaCl/5 mM-sodium acetate (pH 4.0), and 6 M-urea in Krebs-Ringer phosphate buffer (pH 7.4).

	nmol/mg
SH content measured at pH 7.4	0.0
SH content measured at pH 4.0	0.4
SH content measured in 6 M urea, pH 7.4	0.0
Calculated cysteine content	0

	nmol/mg
Cysteic acid content after performic acid oxidation	74
Calculated cystine content	37

Molecular weight of proteoglycan corresponding to one cystine residue per molecule: 27 000 daltons.

Table 2. Amino acid composition of fraction A and B.

The fractions were obtained by gel chromatography on Sepharose 4 B (compare with Fig. 2). The amino acid composition is expressed as residues/1000 residues.

	A	B
Asx	140	153
Thr	38	35
Ser	67	74
Glx	102	106
Pro	62	62
Gly	46	48
Ala	45	43
Cys	29	18
Val	53	52
Met	6	11
Ile	52	47
Leu	154	158
Tyr	38	40
Phe	36	34
Lys	67	63
His	24	21
Arg	32	27

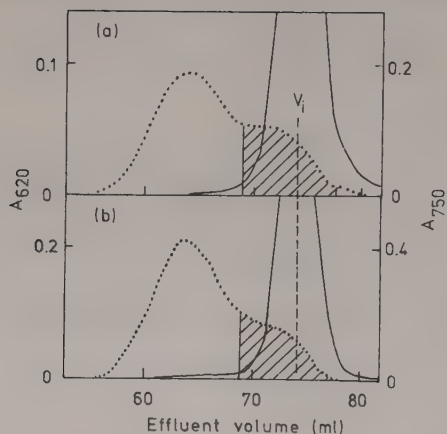


Fig. 11. Chromatography on Sepharose 4B of papain-digested proteoglycan fractions.

Fractions A and B were obtained from a run on a preparative Sepharose 4B column (compare with Fig. 2). An analytical Sepharose 4B column (bed size 0.86 cm x 128 cm, fraction size 1.25 ml) was eluted with 0.5 M sodium acetate (pH 7.0) and analysed for hexose (anthrone, A_{620}) and for protein (Folin, A_{750}). The fractions in the shaded areas were pooled, dialysed, freeze-dried, separated into oligosaccharides and keratan sulphate on ecteola-cellulose microcolumns and analysed for hexosamine.

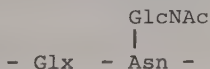
(a) Fraction A. Oligosaccharide-bound hexosamine to keratan sulphate-bound hexosamine ratio: 38:62.

(b) Fraction B. Ratio as in (a): 48:52.

amino acids; only the performic acid method used in this investigation gives reliable values. The other amino acids show the same levels in 70PA and 70PB (Table 2) and in the starting material (Axelsson & Heinegård, 1975). In summary, no certain differences in the composition of 70PA and 70PB could be demonstrated by chemical analyses.

Degradation of the proteoglycans.

The amino acid composition of keratan sulphate peptides purified by ecteola-cellulose chromatography of papain-digested proteoglycans was determined (Table 3). Asparagine has been established as the amino acid to which the N-acetyl-glucosamine of the keratan sulphate chain is bound (Baker et al., 1969 and 1975; Stuhlsatz et al., 1971). Our preparation contains equal amounts of asparagine (and/or aspartic acid) and glutamine (and/or glutamic acid). It seems thus possible that there is one glutamine or glutamic acid residue adjacent to each asparagine residue in the linkage region. It is also possible that there are several glx residues in some linkage regions but none in others. Other amino acids are present in much smaller amounts. The sequence



has been isolated from human IgM and sheep pituitary luteinizing hormone (Marshall, 1972). Corneal keratan sulphate may have a similar structure in the linkage region.

Table 3. Amino acid composition of keratan sulphate peptides

The amino acids are arranged after decreasing abundancy. Values are expressed as residues per 1000 residues.

Asx	223
Glx	221
Ser	86
Leu	79
Gly	75
Pro	51
Cys	46
Thr	38
Ala	34
Tyr	33
Ile	26
Phe	24
Lys	24
Val	19
Met	14
His	5
Arg	Traces

Leucine is the predominant amino acid in the proteoglycans (Axelsson & Heinegård, 1975). The low content of leucine and of other amino acids with hydrophobic side chains (isoleucine, valine) (Table 3) in the keratan sulphate peptides suggests that the non-polar amino acids are uncommon in the vicinity of the keratan sulphate chains.

A considerable fraction (about 20 %) of the hexosamine in corneal proteoglycan preparations is not found in glycosaminoglycan but in neutral or weakly acidic glycans or oligosaccharides, i.e. after digestion with proteases glycopeptides containing 20 % of the total hexosamine appear in a fraction which does not bind to ecteola-cellulose resin at neutral or weakly acidic conditions (Axelsson & Heinegård, 1975). These glycopeptides are partly included in Sephadex G-50 gels (Fig. 12). This means that their molecular size is small and oligosaccharide is probably a more adequate term than glycan. Two questions arise. Firstly, are these oligosaccharides covalently bound to the proteoglycan molecules? Secondly, are these oligosaccharides low-sulphated, low molecular weight types of glycosaminoglycans or are they completely different oligosaccharides? Enzymic degradation experiments indicate that corneal chondroitin sulphate proteoglycans contain oligosaccharides covalently bound to the protein cores of the proteoglycans (unpublished observations). In the case of keratan sulphate proteo-

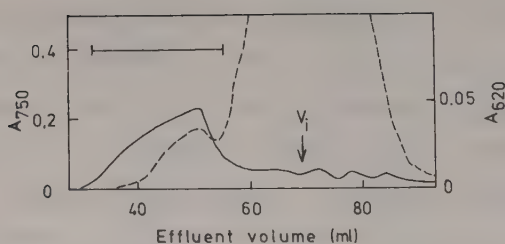


Fig. 12 Chromatography of oligosaccharide peptides on Sephadex G-50 superfine.

The column (bed size 0.8 cm x 138 cm, fraction size 1.6 ml) was eluted with a pyridinium acetate buffer (0.25 M-pyridin solution adjusted with acetic acid to Ph 6.5). The fractions were analysed for hexose (anthrone, A_{620}) and protein (Folin, A_{750}). The fractions indicated by the horizontal bar were pooled and analysed as described in the text.

glycans there are no such direct evidences. It has not been possible, however, for us to separate the oligosaccharides from the proteoglycans by gel chromatography in various buffers, e.g. 6 M-guanidinium chloride or 1 % SDS, or density gradient centrifugation in a solution of CsCl and 4 M-guanidinium chloride (Axelsson & Heinegård, 1975; Axelsson & Heinegård, unpublished). However, it is possible that glycopeptides and proteoglycans are separated by SDS-gel electrophoresis (Fig. 4b). In conclusion, the oligosaccharides are closely associated with the proteoglycans and cannot be removed by the dissociative solvents tested.

In order to determine their composition, oligosaccharide-peptides were isolated after papain digestion of the proteoglycan preparation by means of chromatography on an ecteola-cellulose column and a Sephadex G-50 superfine column as described in the experimental section. The anthrone-positive peak was pooled (Fig. 12). The isolated oligosaccharide-peptides contained the following predominant constituents: Mannose, galactose, glucosamine and aspartic acid/asparagine (molar ratio 2.1: 1.9: 1.5: 1.0; Table 4). Stuhlsatz et al. (1971) have proposed the following structure for corneal keratan sulphate: $(\text{GlcNAc-Gal}(\text{SO}_4))_n\text{-Man-Man-GlcNAc-Asn}$. A mixture of the oligosaccharides $\text{Gal-GlcNAc-Gal-Man-Man-GlcNAc-Asn}$, $\text{GlcNAc-Gal-Man-Man-GlcNAc-Asn}$ and $\text{Gal-Man-Man-GlcNAc-Asn}$ could give molar ratios close to those found in our prepara-

Table 4. Composition of the oligosaccharide peptides isolated from papain-digested keratan sulphate proteglycans.

Values are expressed in $\mu\text{mol/g}$. It was not possible to obtain a reliable value for the xylose content (see the text).

Amino acids

Asx	324
Thr	117
Ser	73
Glx	181
Pro	162
Gly	66
Ala	58
Cys	0
Val	37
Met	5
Ile	91
Leu	144
Tyr	19
Phe	20
Lys	75
His	6
Arg	Traces

Hexosamines

Glucosamine	483
Galactosamine	34

Neutral sugars

Fucose	79
Xylose	<130
Arabinose	Traces
Mannose	694
Galactose	601
Glucose	204

Uronic acids Not detectable

Sialic acids Not analysed

tion. The slight excess of galactose and the presence of some xylose may be due to oligosaccharides with a structure similar to that of the linkage region of corneal galactosaminoglycans which have the following structure (Stuhlsatz et al., 1971): $(\text{GalNac}(\text{SO}_4)_n\text{-UA})_n\text{-Gal-Gal-Xyl-Ser}$. The rather low content of serine suggests that the latter type of oligosaccharides cannot be a major component of the oligosaccharide fraction. It was not possible to obtain a reliable value for the xylose content since the samples contained an unidentified compound which did not separate completely from xylose in the system used for g.l.c. This compound was not eliminated when the sample was purified by AG 1x8 ion exchange chromatography. It was not present in intervertebral disc samples analysed parallel with the corneal samples but appeared again when the whole analytical procedure was repeated. Glucose is at least partly a contamination from the cellulose ion exchange column (Axelsson & Heinegård, 1975).

In sum, the oligosaccharide-peptide fraction has a composition similar to a mixture of corneal glycosaminoglycan linkage regions; it may represent such a mixture. The low sulphated keratan sulphate oligosaccharides from pig cornea described in a preliminary communication by Hirzel et al. (1976) and the structural glycoproteins isolated from calf cornea by Moczar et al. (1969) show similar analytical results with mannose

and glucosamine as predominant sugars.

Tentative model for corneal keratan sulphate proteoglycans.

Corneal keratan sulphate proteoglycans seem to consist of monomers with molecular weights around 72000 and Stokes' radii around 7-12 nm and polydisperse larger molecules with Stokes' radii above 10 nm and similar chemical composition as the monomers. From analytical data (Axelsson & Heinegård, 1975, Table 1, and this paper, Table 4) and value for moisture content (5-10 %; see above) it may be roughly estimated that the proteoglycans consist of about 45 % protein, 30 % keratan sulphate, and 10-12 % oligosaccharides; the rest is probably mostly counter ions. Thus, the average molecular weight of the protein core is about 32000, the total molecular weights of the keratan sulphate chains and the oligosaccharides are about 22000 and about 8000, respectively, in an average proteoglycan molecule. Experiments described above suggest that the S-S-bridges are intrachain; no subunits of the proteoglycan could be identified after reduction indicating that there is only one protein core. The molecular weight of bovine corneal keratan sulphate has been estimated to vary between 4000 and 20000 with most of the chains distributed between 9000 and 19000 (Laurent & Anseth, 1961; Grelling & Stuhlsatz, 1966). The average keratan sulphate pro

teoglycan would then contain one, two or three keratan sulphate chains but there may also be some proteoglycans with several chains since there is a wide variation of the molecular weights of both proteoglycans and keratan sulphate chains. The oligosaccharide structure discussed above have molecular weights around 700-1000; a very rough estimate of the number of oligosaccharide chains in the average molecule is thus less than twelve. It should be reemphasized that the oligosaccharides are closely associated with the proteoglycans but not proved to be covalently linked to them.

Bettelheim and Plessy (1975) have used methods similar to our methods (Antonopoulos et al., 1974) for isolation of corneal proteoglycans. Their recovery of proteoglycans was only 16 per cent of our recovery, probably due to omission of urea from the dialysis and ion exchange chromatography buffers. Despite this, our data are in good agreement with their data. They did not isolate pure keratan sulphate proteoglycans free from other glycosaminoglycans but they obtained two proteoglycan fractions with more keratan sulphate than chondroitin sulphate. The weight-average molecular weight of these fractions were 57000 and 90000 and the partial specific volume was 0.68 ml/g. The only proteoglycans that have been carefully characterized earlier are proteoglycans from cartilage and intervertebral discs. All the data in this paper on the size, molecular weight and composition of the proteoglycans are nothing

but very approximative average values since the proteoglycans show considerable polydispersity and heterogeneity. However, the present investigation clearly shows that corneal keratan sulphate proteoglycans are very different from skeletal proteoglycans: The molecular weight, molecular size and number of glycan chains are only a few per cent of the corresponding values for skeletal proteoglycans; there are considerable amounts of oligosaccharides closely associated with corneal proteoglycans while skeletal proteoglycans are free from oligosaccharides; the protein content is much higher in corneal proteoglycans than in skeletal proteoglycans. This investigation deals with one fourth to one third of the total proteoglycan population of cornea but unpublished results show that the statements made above are true also for the other corneal proteoglycan fractions.

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Foot-note

x) Abbreviations: Gal, galactose; GalNAc, N-acetyl-galactosamine; GlcNAc, N-acetyl-glucosamine; GlcNH₂, glucosamine; Man, mannose; SDS, sodium dodecyl sulphate; UA, uronic acid; V₀, void volume of the gel; V_i, total solvent volume in the gel; Xyl; xylose.

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